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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371**

001560-417

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)

To be assigned

10/069381

INTERNATIONAL APPLICATION NO.
PCT/JP00/05772INTERNATIONAL FILING DATE
25 August 2000PRIORITY DATE CLAIMED
26 August 1999

TITLE OF INVENTION

GENE CODING FOR PROTEIN INVOLVED IN CYTOKININ SIGNAL TRANSDUCTION

APPLICANT(S) FOR DO/EO/US

Tatsuo KAKIMOTO

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☒ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:

PCT Request (Japanese)

PCT Notice Informing the Applicant of the Communication of the International Application to the Designated Offices (Form PCT/IB/308)

Cover page of PCT published application WO 01/16332

PCT International Search Report

International Preliminary Examination Report (Forms PCT/IPEA/46 and PCT/IPEA/409) (Japanese)

Associate Power of Attorney



21839

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)
To be assigned

107069381

INTERNATIONAL APPLICATION NO.
PCT/JP00/05772ATTORNEY'S DOCKET NUMBER
001560-41721. ☒ The following fees are submitted:

CALCULATIONS

PTO USE ONLY

Basic National Fee (37 CFR 1.492(a)(1)-(5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1,040.00 (960)

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00 (970)

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00 (958)

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00 (956)

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 (962)

ENTER APPROPRIATE BASIC FEE AMOUNT =

\$ 890.00

Surcharge of \$130.00 (154) for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492(e)).

20 ☐ 30 ☐

\$

Claims

Number Filed

Number Extra

Rate

Total Claims

40 -20 =

20

X\$18.00 (966)

\$ 360.00

Independent Claims

4 -3 =

1

X\$84.00 (964)

\$ 84.00

Multiple dependent claim(s) (if applicable)

+ \$280.00 (968)

\$

TOTAL OF ABOVE CALCULATIONS =

\$ 1,334.00

Reduction for 1/2 for filing by small entity, if applicable (see below).

+

\$

SUBTOTAL =

\$ 1,334.00

Processing fee of \$130.00 (156) for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492(f)).

20 ☐ 30 ☐

+

\$

TOTAL NATIONAL FEE =

\$ 1,334.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 (581) per property

+

\$ 40.00

TOTAL FEES ENCLOSED =

\$ 1,374.00

Amount to be refunded:

\$

charged:

\$

a. ☐ Small entity status is hereby claimed.b. ☒ A check in the amount of \$ 1,374.00 to cover the above fees is enclosed.c. ☐ Please charge my Deposit Account No. 02-4800 in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed.d. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4800. A duplicate copy of this sheet is enclosed.**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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36,607

REGISTRATION NUMBER

February 26, 2002

DATE

[illegible]

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12

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100

558

Document Describing Recording Format of Flexible Disk etc.

- 1 Name of Applicant Suntory Limited
- 2 Name of Attorney Takashi Ishida
- 3 Indication of International Application
International Application filed on August 25, 2000
Reference No. H782-PCT
- 4 Title of Invention GENE CODING FOR PROTEIN INVOLVED IN
CYTOKININ SIGNAL TRANSDUCTION
- 5 Letter code used Sift JIS code
- 6 Name of file recording sequence
H782-PCT. TXT
- 7 Address for communication
 - Tel No 03(5470)1900
 - Name Tsumoru Fukumoto

20250220 18:06:00

Patent
Attorney's Docket No. 001560-417

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)	
)	
Tatsuo KAKIMOTO)	Group Art Unit: To be assigned
)	
Application No.: National Stage of)	Examiner: To be assigned
PCT/JP00/05772, filed August 25, 2000)	
)	
Filed: February 26, 2002)	
)	
For: GENE CODING FOR PROTEIN)	
INVOLVED IN CYTOKININ)	
SIGNAL TRANSDUCTION)	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to the examination of the above-identified patent application, please enter the following amendments.

IN THE CLAIMS

Please amend Claims 1-5 and 7-12.

1. (Amended) A gene coding for a protein having the amino acid sequence of
SEQ ID NO:2.

2. (Amended) A gene coding for a protein having the amino acid sequence of
SEQ ID NO:2 modified with one or more amino acid additions, deletions and/or
substitutions with other amino acids, wherein said protein is involved in cytokinin signal
transduction.

3. (Amended) A gene which hybridizes with nucleic acid having the nucleotide sequence of SEQ ID NO: 1 or a portion thereof, under stringent conditions and which codes for a protein involved in cytokinin signal transduction.

4. (Amended) A gene according to claim 1, wherein said protein is a histidine protein kinase.

5. (Amended) A vector containing a gene according to claim 1.

7. (Amended) A protein encoded by a gene according to claim 1.

8. (Amended) A method of producing a protein with histidine protein kinase activity, wherein a host according to claim 6 is cultured or cultivated and said protein is obtained from said host.

9. (Amended) A plant or plant cells having introduced therein a gene according to claim 1.

10. (Amended) A method of regulating growth of a plant or plant cells, whereby a gene according to claim 1 is introduced into a plant or plant cells and said gene is expressed.

11. (Amended) A method of inducing adventitious shoot formation in a plant or plant cells, whereby a gene according to claim 1 is introduced into a plant or plant cells and said gene is expressed.

12. (Amended) A method of regulating the floral formation period of a plant, whereby a gene according to claim 1 is introduced into a plant or plant cells and said gene is expressed.

Please add new claims 13-40 as follows:

13. (New) A gene coding for a protein having essentially the amino acid sequence of SEQ ID NO:2, and involved in cytokinin signal transduction.

14. (New) A gene according to claim 2, wherein said protein is a histidine protein kinase.

15. (New) A gene according to claim 3, wherein said protein is a histidine protein kinase.

16. (New) A vector containing a gene according to claim 2.

17. (New) A vector containing a gene according to claim 3.

18. (New) A vector containing a gene according to claim 4.
19. (New) A host transformed by a vector according to claim 16.
20. (New) A host transformed by a vector containing a gene according to claim 17.
21. (New) A host transformed by a vector containing a gene according to claim 18.
22. (New) A protein encoded by a gene according to claim 2.
23. (New) A protein encoded by a gene according to claim 3.
24. (New) A protein encoded by a gene according to claim 4.
25. (New) A protein encoded by a gene according to claim 13.
26. (New) A method of producing a protein with histidine protein kinase activity, wherein a host according to claim 19 is cultured or cultivated and said protein is obtained from said host.

27. (New) A method of producing a protein with histidine protein kinase activity, wherein a host according to claim 20 is cultured or cultivated and said protein is obtained from said host.

28. (New) A method of producing a protein with histidine protein kinase activity, wherein a host according to claim 21 is cultured or cultivated and said protein is obtained from said host.

29. (New) A plant or plant cells having introduced therein a gene according to claim 2.

30. (New) A plant or plant cells having introduced therein a gene according to claim 3.

31. (New) A plant or plant cells having introduced therein a gene according to claim 4.

32. (New) A plant or plant cells having introduced therein a gene according to claim 13.

33. (New) A method of regulating growth of a plant or plant cells, whereby a gene according to claim 2 is introduced into a plant or plant cells and said gene is expressed.

34. (New) A method of regulating growth of a plant or plant cells, whereby a gene according to claim 3 is introduced into a plant or plant cells and said gene is expressed.

35. (New) A method of regulating growth of a plant or plant cells, whereby a gene according to claim 4 is introduced into a plant or plant cells and said gene is expressed.

36. (New) A method of regulating growth of a plant or plant cells, whereby a gene according to claim 13 is introduced into a plant or plant cells and said gene is expressed.

37. (New) A method of regulating the Horal formation period of a plant, whereby a gene according to claim 2 is introduced into a plant or plant cells and said gene is expressed.

38. (New) A method of regulating the Horal formation period of a plant, whereby a gene according to claim 3 is introduced into a plant or plant cells and said gene is expressed.

39. (New) A method of regulating the Horal formation period of a plant, whereby a gene according to claim 4 is introduced into a plant or plant cells and said gene is expressed.

40. (New) A method of regulating the Horal formation period of a plant, whereby a gene according to claim 13 is introduced into a plant or plant cells and said gene is expressed.

REMARKS

Prior to examination of the above-identified application, entry of the foregoing, and consideration of the above amendments are respectfully requested.

The claims have been amended to delete any multiple dependency, and add new claims to cover those embodiments. It is respectfully submitted that no new matter is presented by the above amendments.

Favorable action in the form of a Notice of Allowance is respectfully requested.

In the event that there are any questions relating to this amendment, or to the application in general, it would be appreciated if the Examiner would telephone the undersigned attorney at 508-339-3684 concerning such questions so that prosecution of this application may be expedited.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: Michael K. Meuth #39,300
for Donna M. Meuth
Registration No. 36,607

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Date: February 26, 2002

Attachment to Preliminary Amendment dated February 26, 2002

Marked-up Claims 1-5 and 7-12

1. (Amended) A gene coding for a protein having the amino acid sequence of SEQ ID NO:2 [listed as SEQ. ID. No.2, and involved in cytokinin signal transduction].
2. (Amended) A gene coding for a protein having the amino acid sequence of SEQ ID NO:2 [listed as SEQ. ID. No.2] modified with one or more amino acid additions, deletions and/or substitutions with other amino acids, wherein said protein is [and] involved in cytokinin signal transduction.
3. (Amended) A gene which hybridizes with nucleic acid having the nucleotide sequence [listed as SEQ. ID. No.2] of SEQ ID NO: 1 or a portion thereof, under stringent conditions and which codes for a protein involved in cytokinin signal transduction.
4. (Amended) A gene according to claim 1 [any one of claims 1 to 3], wherein said protein is a histidine protein kinase.
5. (Amended) A vector containing a gene according to claim 1 [any one of claims 1 to 4].
7. (Amended) A protein encoded by a gene according to claim 1 [any one of claims 1 to 4].

Attachment to Preliminary Amendment dated February 26, 2002

Marked-up Claims 1-5 and 7-12

8. (Amended) A method of producing a protein with histidine protein kinase activity, wherein [characterized in that] a host according to claim 6 is cultured or cultivated and said protein is obtained from said host.

9. (Amended) A plant or plant cells having introduced therein a gene according to claim 1 [any one of claims 1 to 4].

10. (Amended) A method of regulating growth of a plant or plant cells, whereby a gene according to claim 1 [any one of claims 1 to 4] is introduced into a plant or plant cells and said gene is expressed.

11. (Amended) A method of inducing adventitious shoot formation in a plant or plant cells, whereby a gene according to claim 1 [any one of claims 1 to 4] is introduced into a plant or plant cells and said gene is expressed.

12. (Amended) A method of regulating the floral formation period of a plant, whereby a gene according to claim 1 [any one of claims 1 to 4] is introduced into a plant or plant cells and said gene is expressed.

--ABSTRACT

A gene originating in, for example, *Arabidopsis thaliana*, having an amino acid sequence represented by SEQ ID NO:2, and encoding a protein which participates in the signal transduction of cytokinin. This gene is usable in producing the above protein. By transferring into a plant, moreover, it is usable for the regeneration, differentiation, growth, etc. of the plant.--

DESCRIPTION

GENE CODING FOR PROTEIN INVOLVED IN
CYTOKININ SIGNAL TRANSDUCTION

5

Technical Field

The present invention relates to a gene coding for a protein involved in cytokinin signal transduction, and to a method for its use.

10

Background Art

Cytokinin is the general term for a class of plant hormones that play an important role in plant morphogenesis and physiological phenomena, and a class of derivatives having a basic skeleton with, usually, a 5C substituent bonded to the 6-position amino group of 6-aminopurine, and with various modifications on side chains. Cytokinins interact with auxins, another class of plant hormones, to control plant cell division and differentiation and new organogenesis in tissue culture systems. As for organogenesis, auxins promote formation of roots from calli, leaves and stems, while cytokinins promote bud formation. When cytokinins are given to normal plant bodies, they exhibit such effects as promotion of lateral bud growth, inhibition of root growth, and retardation of flowers, leaves, fruits and the like. When given to seeds, they exhibit an effect of promoting germination, depending on the plant species.

25

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The various physiological effects mentioned above are believed to result from reception of cytokinins by cells, conversion to intracellular chemical signals, transduction of those signals, and control of expression of various target genes located downstream therefrom.

35

While regulation of the cytokinin synthesis pathway is clearly connected with control of the various physiological functions exhibited by cytokinins, little is known about the molecular mechanisms controlling

synthesis of cytokinins in plants.

Phosphate group transfer by a two-component system has recently been clearly implicated in at least a part of the cytokinin signal transduction pathway (Kakimoto, Science, 274, p.982-985, 1996, Kakimoto, Saibo Kagaku [Cell Engineering], Special issue: Shokubutu Saibo Kogaku [Plant Cell Engineering] Series 10, p.75-84, 1998). The two-component system is a type of signal transduction pathway widely found in prokaryotic organisms, and it basically involves two proteins known as a sensor histidine kinase and a response regulator.

Most histidine kinases span the cell membrane at their N-terminal region, with the N-terminal region receiving the external signal, but the majority of cytoplasm-localized histidine kinases perform roles mediated by signals from receptors. When the input domain of the sensor histidine kinase detects a signal, a specific histidine residue of the histidine kinase domain is phosphorylated. The phosphate group is then transferred to a specific aspartic acid residue of the response regulator which is the other protein of the two-component system, and the presence or absence of this phosphorylation leads to the downstream signal transduction pathway and the target gene expression regulation.

In a two-component system, the phosphate group is often first transferred from the sensor histidine kinase to a protein known as a phosphate group transfer mediator, and finally to the response regulator. For example, in the osmoregulatory pathway of yeast (*Saccharomyces cerevisiae*), the phosphate group is transferred from a sensor histidine kinase, osmolarity receptor SLN1, to a phosphate group transfer mediator, YPD1, and is then transferred to a response regulator, SSK1. Prokaryotes are known to possess numerous signal transduction mechanisms via two-component systems, but recently two-component system factors have also been

found to perform important functions in fungi and plants as well. These include, for example, the yeast osmoregulatory pathway and the plant ethylene signal transduction pathway.

5 The present inventor considered that it might be possible to elicit a cytokinin response even in the absence of cytokinins, by increasing the level of transcription of genes involved in cytokinin synthesis or in the signal transduction pathway, and thus carried out
10 activation tagging. This is a method whereby an enhancer sequence is randomly introduced into a plant genome to activate transcription of a plant gene near the inserted enhancer in order to obtain a dominant mutant. The present inventors transformed approximately 50,000
15 *Arabidopsis thaliana* calli using the vector pPCVICEn4HPT (Hayashi et al., Science, 258, p.1350-1353, 1992) developed for activation tagging.

20 pPCVICEn4HPT contains a strong enhancer sequence derived from the enhancer of the cauliflower mosaic virus 35S RNA gene on T-DNA, and after insertion into the plant genome via *Agrobacterium*, transcription of the gene adjacent to the enhancer is activated. For example, if the T-DNA is inserted near a gene involved in a cytokinin
25 synthesis pathway, receptor or signal transduction pathway, the transformant would be expected to exhibit a typical cytokinin response even in the absence of the cytokinin. The present inventor cultured over 50,000 calli in medium containing no plant hormones, and separated out 4 lines of transformed calli which
30 exhibited the typical cytokinin responses of cell growth and adventitious shoot formation.

35 These were designated as cytokinin independent (*ckil*) mutants. They exhibited cell growth, adventitious shoot formation and virescence, but formed no roots and were sterile. The 4 lines had the T-DNA inserted upstream from the same gene, and as a result of analysis of the causative gene *CK11*, the protein coded for by the

CKI1 gene was found to have the characteristic sequence of a sensor histidine kinase of a two-component system, with two hydrophobic domains believed to be membrane spanning domains at the N-terminal end.

5 CKI1 has been demonstrated to elicit a typical cytokinin response when overexpressed in plants (Kakimoto, Science, 274, p.982-985, 1996). Expression of CKI1 gene in yeast have implicated in transfer of a phosphate group to the osmoregulatory two-component
10 system factor YPD1 in yeast. This suggests that CKI1 is a cytokinin receptor with a function of converting cytokinin stimulation into the chemical signal of phosphate transfer, and transmitting it to a downstream factor.

15 In addition, Sakakibara et al. (Plant J., 14, p.337-344, 1998) have reported that two-component system response regulators are encoded by genes such as ZmCip1 whose expression is elicited by treatment of maize leaves with cytokinins, or IBC6 whose expression is elicited by
20 treatment of *Arabidopsis thaliana* with cytokinins, and therefore two-component systems are also thought to be involved in the cytokinin signal transduction pathway. Such findings indicate the possibility that these response regulators act downstream from histidine kinase
25 cytokinin receptors.

 However, the constituent of the cytokinin signal transduction pathway by CKI1 or ZmCip1 proteins remains unclear. For example, it is unknown whether phosphate group transfer mediator is involved in phosphate group
30 transfer from histidine kinase CKI1, as the candidate cytokinin receptor, to the downstream response regulator. Moreover, it is unknown whether or not cytokinin signals are transduced by a plurality of cytokinin receptors in independent signal transduction pathways acting in
35 parallel, and whether or not such a plurality of signal transduction pathways interact with and affect each other, and this remains as an issue to be resolved in the

future.

Disclosure of the Invention

5 It is therefore an object of the present invention to provide a novel factor of a two-component system involved in the cytokinin signal transduction pathway, a gene coding therefor, a protein encoded by the gene, and uses therefor.

10 The present inventor carried out activation tagging using *Arabidopsis thaliana* and obtained a novel gene coding for a two-component system factor involved in the cytokinin signal transduction pathway.

15 The present invention provides, therefore, a gene coding for a protein involved in the cytokinin signal transduction pathway. Specifically, the gene codes for a protein with histidine protein kinase activity in a two-component system. The protein encoded by the gene is predicted to be soluble in the cytoplasm, and no gene coding for a histidine protein kinase with such
20 properties has yet been reported for plants.

More specifically, the invention provides a gene coding for a protein having the amino acid sequence listed as SEQ. ID. No.2 and involved in cytokinin signal transduction. The invention further provides a gene
25 coding for a protein having the amino acid sequence listed as SEQ. ID. No.2 modified with one or more amino acid additions, deletions and/or substitutions with other amino acids, and having activity involved in cytokinin signal transduction. The invention still further
30 provides a gene which hybridizes with the nucleic acid listed as SEQ. ID. No.1, and particularly DNA or a portion thereof, and which codes for a protein involved in cytokinin signal transduction and particularly a protein having histidine protein kinase activity.

35 The invention still further provides a vector containing the aforementioned gene.

The invention still further provides a host

transformed by the aforementioned vector. The host may be plant cells or a plant body.

The invention still further provides a method of producing a protein involved in cytokinin signal transduction by culturing or cultivating the
5 aforementioned host.

The invention still further provides a method of regulating growth of a plant or plant cells by introducing the aforementioned gene into the plant or
10 plant cells and by expressing the gene. In other words, by expressing this gene it is possible to regulate various physiological functions associated with cytokinins, such as adventitious shoot formation promotion, breakage of lateral bud dormancy, retardation
15 of flower and leaf aging and fruit maturation, improvement of flower lifespan and maintenance of photosynthetic function, promotion of fruit enlargement and prevention of fruit abscission, flower formation control, etc.

20

Brief Description of the Drawings

Fig. 1 is an illustration showing the structure of plasmid pBI35T.

Fig. 2 is an illustration showing the structure of
25 plasmid pBI35T.CKI2.163.

Fig. 3 is an illustration showing the structure of plasmid pBI35T.CKI2.162.

Fig. 4 is an illustration showing the structure of plasmid pEL2 omega35T.

Fig. 5 is an illustration showing the structure of
30 plasmid pEL2 omega35T.CKI2.163.

Fig. 6 is an illustration showing the structure of plasmid pEL2 omega35T.CKI2.162.

35

Best Mode for Carrying Out the Invention

The present inventor considered that it might be possible to obtain a mutation introduced plant with a new

protein gene involved in the cytokinin signal transduction pathway by activation tagging in the same manner as when isolating the CKI1 gene. *Arabidopsis thaliana* transformant calli having vector pPCVICen4HPT for activation tagging introduced via *Agrobacterium* were screened on cytokinin-free medium, and transformants that formed adventitious shoots in the absence of cytokinins were separated. Of these, a transformed callus named cki2 exhibited abundant cell growth and formed adventitious shoots in the absence of cytokinins, unlike the wild-type callus.

As a result of isolating and analyzing the CKI2 gene and CKI2 cDNA from the cki2 mutant, it was found that the CKI2 gene codes for a protein homologous with the plant histidine protein kinase of a two-component system. Particularly high homology was found in two functional domains of the histidine protein kinase, in the histidine kinase domain and the receiver domain.

The cki2 mutant phenotype is similar to the publicly known cki1 mutant in the aspect of forming adventitious shoots in the absence of cytokinins, but the overall structures and amino acid sequences of CKI2 and CKI1 proteins are homologous only on a general level for histidine kinases with different functions, and CKI2 and CKI1 do not exhibit particularly high homology.

The predicted protein also differs considerably in terms of its overall structure and intracellular localization.

When a portion of CKI2 cDNA was introduced into *Arabidopsis thaliana* callus and overexpressed, the transformants exhibited active cell growth and adventitious shoot formation independently of cytokinins in the medium, as was also predicted from the cki2 mutant phenotype. This demonstrated that the CKI2 gene codes for a novel histidine protein kinase involved in cytokinin signal transduction.

The gene of the invention includes any one coding

for the amino acid sequence listed as SEQ. ID. No.2, for example. However, it is known that a protein having an amino acid sequence modified with a plurality of amino acid additions, deletions and/or substitutions with other amino acids may maintain similar activity as the original protein. Therefore, the invention also encompasses modified genes coding for proteins having the amino acid sequence listed as SEQ. ID. No.2 modified with one or more amino acid additions, deletions and/or substitutions with other amino acids and having activity involved in cytokinin signal transduction, particularly histidine protein kinase activity.

The degree of modification referred to here is a degree which can be achieved by technical means well known prior to filing of the present application, such as site-specific mutagenesis or PCR. The number of amino acids to be modified while maintaining histidine protein kinase activity may be, for example, no greater than 100, no greater than 50, preferably no greater than 25, and especially no greater than 10.

The invention further provides a gene which can hybridize with nucleic acid, such as DNA, having the nucleotide sequence listed as SEQ. ID. No.1, or a portion thereof, under stringent conditions and which is composed of DNA coding for a protein with histidine protein kinase activity. The stringent conditions referred to here are, for example, conditions of hybridization at 5xSSC, 50°C. The suitable hybridization temperature will depend on the nucleotide sequence and the nucleotide sequence length, and may be selected as appropriate.

The portion of nucleic acid referred to above is a portion coding for at least a few contiguous amino acid sequences, and preferably a portion coding for a few contiguous amino acid sequences in a histidine kinase domain or receiver domain. There may be used, for example, a sequence known to be a well-conserved sequence (Albright et al., Annu. Rev. Genet. 23, p.311-336, 1989;

Mizuno, J. Biochem. 123, p.555-563, 1998). More preferably, it refers to a portion or fragment including all or part of a histidine kinase domain or receiver domain of the sequence listed as SEQ. ID. No.1, and
5 having at least 25%, preferably at least 50% and more preferably at least 75% of the total coding sequence listed as SEQ. ID. No.1.

The gene source used as the object of the hybridization may be a cDNA library or genome DNA library
10 prepared from a plant or microorganism with a protein involved in cytokinin signal transduction, particularly a histidine protein kinase, with examples of such plants including *Arabidopsis thaliana*, maize, poplar, petunia, tobacco, rice, tomato, eucalyptus and the like.

15 The nucleotide sequence of the gene coding for the protein involved in cytokinin signal transduction obtained in this manner has at least 50% or at least 60%, preferably at least 70% or at least 80% and especially at least 90% homology with the nucleotide sequence listed as
20 SEQ. ID. No.1.

The gene coding for a protein with the amino acid sequence listed as SEQ. ID. No.2 may be obtained from *Arabidopsis thaliana* in the form of cDNA or genomic DNA.

A gene with the naturally occurring nucleotide
25 sequence may be obtained by screening of a cDNA library, for example, as explained in detail in the examples. Also, DNA coding for a protein with a modified amino acid sequence may be synthesized by commonly used site-specific mutagenesis or PCR, based on DNA having the
30 naturally occurring nucleotide sequence. For example, a DNA fragment in which the modification is to be introduced may be obtained by restriction enzyme treatment of the naturally occurring cDNA or genomic DNA, and used as a template for site-specific mutagenesis or
35 PCR using primers including the desired mutation, to obtain a DNA fragment with the desired modification introduced therein. The mutation-introduced DNA fragment

may then be linked with a DNA fragment coding for another part of the target protein.

Alternatively, when DNA coding for an amino acid sequence longer than the target amino acid sequence, such as the full length amino acid sequence, is digested with a selected restriction enzyme in order to obtain DNA coding for a protein comprising a shortened amino acid sequence, and the obtained DNA fragment consequently does not code for the entire amino acid sequence, a DNA fragment comprising the missing sequence portion may be synthesized and linked thereto.

By using a gene expression system to express the obtained gene in *E. coli* or yeast, it is possible to obtain a histidine protein kinase as the gene product. Alternatively, a histidine protein kinase may also be obtained using antibodies for a protein having the amino acid sequence listed as SEQ. ID. No.2, and such antibodies may be used to clone a histidine protein kinase of another organism.

Thus, the present invention also relates to a recombinant vector, and particularly an expression vector, containing the aforementioned gene, and to a host transformed by the vector. The host used may be a prokaryotic or eukaryotic organism. As prokaryotic organisms there may be mentioned commonly used host microorganisms including bacteria, for example, bacteria belonging to the genus *Escherichia* such as *Escherichia coli*, and microorganisms belonging to the genus *Bacillus* such as *Bacillus subtilis*.

As eukaryotic hosts there may be used lower eukaryotes, for example, eukaryotic microorganisms such as yeast and fungi. As yeasts there may be mentioned, for example, microorganisms belonging to the genus *Saccharomyces*, such as *Saccharomyces cerevisiae*, and as fungi there may be mentioned microorganisms belonging to the genus *Aspergillus*, such as *Aspergillus oryzae* and *Aspergillus niger*, and microorganisms belonging to the

genus *Penicillium*. Animal cells or plant cells may also be used, and animal cells which may be used include mouse, hamster, monkey and human cell systems. Plant cells which may be used include cultured cells of tobacco, and cultured cells of *Populus*, *Eucalyptus* and *Acacia*.

Insect cells such as silkworm cells, or even the silkworm imago itself, may be used as the host. Specifically, there may be used insect cells such as *Spodoptera frugiperda* cells, *Trichoplusia ni* cells or *Bombyx mori* cells and animals cells such as human cells, monkey cells or mouse cells, and more specifically COS cells, Vero cells, CHO cells, L cells, C127 cells, BALB/c 3T3 cells, Sp-2/0 cells and the like.

The expression vector used may be plasmid, phage, phagemid, virus (baculovirus (insect) or vaccinia (animal cells), or the like.

The expression vector of the invention will contain expression control regions, such as a promoter and terminator, replication origin, etc. depending on the type of host into which it is to be introduced. The promoter used for a bacterial expression vector may be a common promoter such as *trc* promoter, *tac* promoter, *lac* promoter or the like, the promoter used for yeast may be, for example, glyceraldehyde 3-phosphate dehydrogenase promoter, PH05 promoter, *adhI* promoter, *pqk* promoter or the like, and the promoter used for fungi may be, for example, *amy* promoter, *trpC* promoter or the like.

As promoters for insects there may be mentioned baculovirus polyhedrin promoter, and as promoters for animal cells there may be mentioned Simian Virus 40 early and late promoters, CMV promoter, HSV-TK promoter or SR α promoter or the like. As promoters for plants there may be mentioned cauliflower mosaic virus 35S promoter and nopaline synthase promoter, and as induction promoters there may be mentioned glutathione-S-transferase II gene promoter, hsp80 promoter, ribulose 2-phosphate

carboxylase small subunit gene promoter, and the like.

The expression vector is preferably in a form including not only the above, but also an enhancer, splicing signal, polyA addition signal, selectable marker (for example, a dihydrofolate reductase gene (methotrexate resistance), neo gene (G418 resistance), etc.) and the like. When an enhancer is used, for example, an SV40 enhancer, it may be inserted either upstream or downstream from the gene.

The transformation of the host by the expression vector may be accomplished by an ordinary method well known to those skilled in the art, and such methods are described, for example, in Current Protocols in Molecular Biology, John Wiley & Sons, 1995. The transformants may also be cultured by an ordinary method. Purification of the protein involved in cytokinin signal transduction from the cultured product may be accomplished according to ordinary methods for isolation and purification of proteins, such as ultrafiltration, various column chromatography methods (chromatography using Sepharose, etc.), and the like.

With the current level of technology, the cDNA or gene may be linked under the control of a constitutive or inducible promoter, and the gene may be introduced into and expressed in a plant by a system employing *Agrobacterium* or a particle gun, electroporation or the like, to promote adventitious shoot formation in plants such as roses which are resistant to individual regeneration even by artificial regulation with plant hormones. In addition, by controlling expression of the CKI2 gene it is possible to regulate various physiological effects exhibited by cytokinins in plants, such as lateral bud growth, retardation of aging, flower formation, promotion of fruit enlargement and prevention of fruit abscission.

Examples

The invention will now be explained in greater detail by way of examples. Unless otherwise specified, the molecular biological methods used were based on Molecular Cloning (Sambrook et al., 1989).

5 Example 1 Screening of cytokinin-responding mutants

Activation tagging was carried out using *Arabidopsis thaliana* Wassileuskiya (WS ecotype) in order to increase transcription of genes involved in the cytokinin synthesis or signal transduction pathway and obtain
10 mutants exhibiting a cytokinin response even in the absence of cytokinins.

The activation tagging vector pPCVICEn4HPT (Hayashi et al., Science, 258, p.1350-1353, 1992) was used for transformation of over 50,000 *Arabidopsis thaliana* WS
15 ecotype calli according to the method of Akama et al. (Akama et al., Plant Cell Rep., 12, 7-11, 1992). pPCVICEn4HPT contains a strong enhancer sequence derived from the cauliflower mosaic virus 35S RNA gene promoter, and after insertion into the plant genome, transcription
20 of the gene adjacent to the enhancer sequence is activated.

The transformed calli were cultured on medium containing no plant hormones. Cell growth of the wild-type *Arabidopsis thaliana* calli is inhibited on medium
25 containing no cytokinins such that adventitious shoots cannot be formed, but adventitious shoots are formed in the presence of cytokinins. Among the transformants, however, there were obtained calli which exhibited a typical cytokinin response and formed adventitious shoots
30 even though no cytokinins were present.

Among these, 4 lines designated as ckil-1, ckil-2, ckil-3 and ckil-4 resulted from overexpression of the same gene, and the causative gene CKI1 has already been reported by the present inventor (Kakimoto, Science,
35 274:982-985, 1996). In addition to these 4 ckil lines, there was also obtained a phenotype similar to ckil, i.e. the mutant cki2 having a different causative gene than

the causative gene for ckil, while also clearly exhibiting more abundant cell growth than the wild-type callus and exhibiting adventitious shoot formation, in the absence of cytokinins.

5 Example 2 Isolation of causative gene of cki2 mutant

Genomic DNA was purified from the cki2 mutant, and after digestion with restriction enzyme SphI, T4 DNA ligase was used for self circularization. This was
10 introduced into *E. coli* and the plasmid was purified from the ampicillin-resistant colonies. The plasmid contains the T-DNA and the DNA sequence adjacent to the T-DNA right border (RB) in the genome of the cki2 mutant.

Oligotex dT-30 (Nihon Roche) was used to purify mRNA
15 from RNA extracted from the cki2 mutant and the entire plant body of a wild-type *Arabidopsis thaliana* WS ecotype, and this was used as the template to create a cDNA library using a Lambda ZAPII cDNA library synthesis kit (Stratagene Co.) by the method recommended by
20 Stratagene Co. The cki2 mutant and wild-type *Arabidopsis thaliana* WS ecotype cDNA libraries were screened using as a probe the genomic sequence adjacent to the RB of the cki2 mutant obtained as described above.

The nucleotide sequence of the CKI2 cDNA obtained
25 from the cDNA library of the wild-type *Arabidopsis thaliana* WS ecotype was determined, and is listed as SEQ. ID. No.1 of the Sequence Listing. The amino acid sequence encoded thereby is listed as SEQ. ID. No.2. The DNA codes for an amino acid sequence homologous with that
30 of a two-component system histidine protein kinase, and particularly it was shown to have high homology with histidine kinase domains and receiver domains which are the two histidine protein kinase functional domains. For example, identical amino acid residues constitute 35% in
35 the histidine kinase domain and 25% in the receiver domain of the sensor histidine kinase ETR1 which is believed to be an ethylene receptor, 41% in the histidine

kinase domain and 26% in the receiver domain of the
sensor histidine kinase Lema of the prokaryote
Pseudomonas, and 32% in the histidine kinase domain and
26% in the receiver domain of CKI1 reported by the
5 present inventor.

The amino acid residues that are well conserved
among most two-component system factors were also well
conserved in CKI2. However, the sequence of
approximately 360 amino acids at the N-terminus of CKI2
10 protein has not been found to have significant homology
with any known sequence. Moreover, although a membrane-
spanning domain is present at the N-terminus of the
sensor histidine kinases ETR1 and CKI1, the hydrophobic
regions characteristic of membrane-spanning domains were
15 not found at the N-terminus of CKI2 protein. The CKI2
gene product is therefore predicted to reside in the
cytoplasm.

As a result of determining the nucleotide sequence
of the cDNA obtained from the cki2 mutant cDNA library
20 and comparing it with the nucleotide sequence of the CKI2
cDNA obtained from the aforementioned wild-type cDNA
library, it was shown that in the cki2 mutant, T-DNA was
inserted between bases 249 and 250 from the 5' end of the
coding region of CKI2 (counting the A of ATG of the
25 translation initiation codon as base 1), and that
transcription had begun from within the enhancer sequence
on the T-DNA. This indicated that a transcription
product of the CKI2 gene lacking the 5' end had been
produced in the cki2 mutant, and a CKI2 protein lacking
30 the N-terminus had thus been synthesized. It was
surmised that the cki2 mutant exhibited a cytokinin
response even in the absence of cytokinins because of
constitutive production of the N-terminus lacking CKI2
protein due to the enhancer sequence.

35 The genomic sequence of the CKI2 gene of *Arabidopsis*
thaliana WS ecotype was determined in the following
manner. First, total genomic DNA of *Arabidopsis thaliana*

WS ecotype was used as a template for amplification by PCR (using LA Taq (Takara), with reaction conditions of 1 minute at 96°C followed by 1 minute at 96°C, 1 minute at 52°C and 3 minutes at 68°C repeated for 4 cycles, and then 1 minute at 96°C, 1 minute at 57°C and 3 minutes at 68°C repeated for 26 cycles, and finally 10 minutes of reaction at 68°C) using primer No.163 (5'-CGCGGATCCACCATGGTCTGTGAAATGGAGAC-3') (SEQ. ID. No.7) and primer No.154 (5'-CCGCTCGAGTCAGTGCAAATACTGTTGCAAAC-3') (SEQ. ID. No.8), and the nucleotide sequence of the coding region of the amplified CKI2 gene was determined.

Genomic DNA of the cki2 mutant was digested with BamHI, and T4-DNA ligase was used for self circularization. This was introduced into *E. coli* DH10B, and the plasmid was purified from *E. coli* of the colonies which exhibited ampicillin resistance. The plasmid contained a DNA sequence adjacent to the T-DNA left border (LB) in the genome of the cki2 mutant. The DNA adjacent to the LB also contained a sequence believed to include the coding region upstream from the T-DNA insertion point and the promoter further upstream from it, of the CKI2 gene. A portion of the nucleotide sequence of this plasmid was determined, and by combining it with the CKI2 cDNA sequence listed as SEQ. ID. No.1, the genomic sequence including the CKI2 coding region and its upstream sequence was determined. This is listed as SEQ. ID. No.3. The amino acid sequence encoded thereby is listed as SEQ. ID. No.4.

PCR was then conducted (using LA Taq (Takara), with 1 minute at 94°C, 30 seconds at 94°C, 30 seconds at 52°C and 3 minutes at 68°C for 30 cycles, and then 7 minutes reaction at 68°C) with *Arabidopsis thaliana* Landsberg erecta (Ler) strain, using primer No.163 and primer No.177 (5'-GGGGTACCTCAGTGCAAATACTGTTGCAAAC-3') (SEQ. ID. No.9), and the nucleotide sequence of the amplified DNA was determined. The sequence was compared with the nucleotide sequence of the CKI2 coding region of WS

ecotype and found to be 99.6% identical thereto, and the sequence was therefore designated as the CKI2 gene sequence for Ler strain.

5 The nucleotide sequence of the CKI2 genome gene of *Arabidopsis thaliana* Ler strain is listed as SEQ. ID. No.5. The protein amino acid sequences deduced from the expected exon sequences of *Arabidopsis thaliana* Ler strain and WS ecotype differed in three amino acid residues. The amino acid sequence encoded thereby is
10 listed as SEQ. ID. No.6.

Example 3 Overexpression of CKI2 gene

1) Enhancement of transforming binary vector

pBI121 (Jefferson, R.A. et al., EMBO J., 6:3901-
15 3907, 1987), which is widely used as a binary vector for plant transformation, has the β -glucuronidase (GUS) gene positioned between the cauliflower mosaic virus 35S RNA gene promoter (35S promoter) and the nopaline synthase (NOS) gene terminator in the T-DNA region.

20 Also, pBE2113-GUS (Mitsuhara, I. et al., Plant Cell Physiol., 37:49-59, 1996) is based on pBI121 and has an added enhancer sequence for reinforced promoter activity and an added omega translation enhancing sequence.

In order to use pBI121 or pBE2113-GUS for
25 overexpression of the specific gene in plants, two types of restriction enzymes are used to remove the GUS gene, and the specific gene is cloned instead at that section. However, since of the enzymes used to remove the GUS gene, only SacI can be used as the enzyme for digesting
30 of the 3' end of the GUS gene in both pBI121 and pBE2113GUS, it was decided to insert a new cloning site.

The NOS gene terminator sequence is present at two locations in both pBI121 and pBE2113-GUS, and the presence of the same sequence in one vector entails the
35 risk of lower stability of the T-DNA in *Agrobacterium* or in the transformed plant. The GUS gene and the NOS gene terminator sequence downstream therefrom were therefore

removed from each of the vectors, and the cauliflower mosaic virus 35S RNA gene terminator (35S terminator) was inserted in their place.

5 Plasmid p1'barbi (Mengiste, T., Plant J., 12:945-948, 1997) was used as a template for amplification (using LA Taq (Takara), with 1 minute at 94°C, 30 seconds at 94°C, 30 seconds at 52°C and 30 seconds at 68°C for 15 cycles, and then 3 minutes reaction at 68°C) using primer No.271 (5'-TACCCGGGGGTACCGTCGACCTGCAGGCATGCC-3') (SEQ. ID. No.10) and primer No.272 (5'-AAACGACGGCCAGTGAATTCGAGTTCGGCACC-3') (SEQ. ID. No.11) as the primers, the amplified DNA (containing the 35S terminator) was treated with SmaI and EcoRI and the resulting DNA fragment was ligated with a DNA fragment obtained from pBI121 by treatment with SmaI and EcoRI to remove the GUS gene and NOS gene terminator, and the product was designated as pBI35T (Fig. 1).

20 In pBI35T, the unique sites of the multicloning sites between the 35S promoter and 35S terminator were XbaI, BamHI, KpnI and SalI from the end near the 35S promoter. Similarly, the DNA fragment obtained by SmaI and EcoRI treatment of DNA (containing the 35S terminator) amplified using primer No.271 and primer No.272 as the primers with p1'barbi as the template was ligated with a pBE2113-GUS derived DNA fragment obtained from pBE2113-GUS by treatment with SmaI and EcoRI to remove the GUS gene and NOS gene terminator, to obtain pEL2omega35T (Fig. 4). The unique sites of the multicloning sites between the reinforced 35S promoter and 35S terminator in pEL2omega35T were XbaI, BamHI, KpnI and SalI from the end near the 35S promoter.

ii) Construction of plasmid for CKI2 gene expression

35 Because growth of *E. coli* containing CKI2 cDNA was poor, cloning downstream from the 35S promoter was expected to be difficult. It was therefore decided to use CKI2 genomic DNA for the experiment of overexpression of all or part of the CKI2 gene in plants. An

Arabidopsis thaliana Ler strain genome library (Fuse, T. et al., Plant J. 7:849-856, 1995) constructed in Lambda TI2 vector was screened and a clone (LambdaTi.CKI2-2) containing the CKI2 gene was isolated.

5 The sequence of the CKI2 coding region of the clone is consistent with SEQ. ID. No.5. LambdaTi.CKI2-2 was used as the template for amplification by PCR (using LA Taq (Takara), with reaction for 1 minute at 94°C, followed by 30 seconds at 94°C, 30 seconds at 50°C and 3
10 minutes at 68°C for 13 cycles, and then 7 minutes reaction at 68°C) with primer No.163 and primer No.177, and cloning was carried out at the restriction enzyme SrfI recognition site of pCR-ScriptSK(+) with blunt ends, to obtain plasmid pCKI2.3LD1. The nucleotide sequence of
15 the CKI2 gene portion of pCKI2.3LD1 matched the nucleotide sequence of the *Arabidopsis thaliana* Ler strain CKI2 gene.

 After digesting pCKI2.3LD1 with KpnI and BamHI, the fragment containing the CKI2 gene was cloned between the
20 KpnI site and BamHI site of pBI35T and between the KpnI site and BamHI site of pEL2omega35T, and these were designated as pBI35T.CKI2.163 (Fig. 2) and pEL2omega35T.CKI2.163 (Fig. 5), respectively. These two clones had the entire coding region of the CKI2 gene
25 cloned in the sense direction with respect to the 35S promoter.

 Also, LambdaTi.CKI2-2 was used as the template for amplification by PCR (using LA Taq (Takara), with reaction for 1 minute at 94°C, followed by 30 seconds at
30 94°C, 30 seconds at 50°C and 3 minutes at 68°C for 13 cycles, and then 7 minutes reaction at 68°C) with primer No.162 (5'-CGCGGATCCACCATGCTTGAGGCGAGTACTG-3') (SEQ. ID. No.12) and primer No.177, and cloning was carried out at the restriction enzyme SrfI recognition site of pCR-ScriptSK(+) with blunt ends, to obtain plasmid
35 pCKI2.2L16. The nucleotide sequence of the CKI2 gene portion of pCKI2.2L16 matched the nucleotide sequence of

the *Arabidopsis thaliana* Ler strain CKI2 gene.

After digesting pCKI2.2L16 with KpnI and BamHI, the fragment containing a part of the CKI2 gene was cloned between the BamHI site and KpnI site of pBI35T and
5 between the BamHI site and KpnI site of pEL2omega35T, and these were designated as pBI35T.CKI2.162 (Fig. 3) and pEL2omega35T.CKI2.162 (Fig. 6), respectively. These two clones coded for the portion from the 178th methionine to the carboxyl end of the protein encoded by the CKI2 gene.

10 The following experiment was conducted to investigate whether or not a cytokinin response is elicited by overexpression of the CKI2 gene as the entire region of the CKI2 protein or as the region after the 178th methionine. *Arabidopsis thaliana* WS ecotype calli
15 were transformed by coculturing with *Agrobacteria* containing pBI35T, pBI35T.CKI2.163, pBI35T.CKI2.162, pEL2omega35T, pEL2omega35T.CKI2.163 or pEL2omega35T.CKI2.162, and were cultured on the following three different solid medium, that is, GM medium (4.32
20 g/l Murashige-Skoog salts (Sigma), 1% sucrose, 10 ml/l 5% 2-(N-morpholino)ethanesulfonic acid (adjusted to pH 5.8 with KOH), 100 mg/l inositol, 10 mg/l thiamin-HCl, 1 mg/l pyridoxine-HCl, 1 mg/l nicotinic acid and 0.3% Phytage ITM (Sigma)) with addition of solid medium Z-0 containing
25 50 mg/l kanamycin sulfate, 100 mg/l cefotaxime, 100 mg/l vancomycin and 0.3 mg/l indoleacetic acid, and further addition of solid medium Z-0.1 containing 0.1 mg/l transzeatin and solid medium Z-1 containing 1 mg/l transzeatin.

30 The calli transformed with pBI35T and the calli transformed with pEL2omega35T exhibited no virescence and formed no shoots (buds) on the Z-0 medium, but exhibited virescence and shoot (bud) formation with low frequency on Z-0.1 medium, whereas virescence and shoot formation
35 were found in over half of the calli on the Z-1 medium. In contrast, almost all of the calli transformed with pBI35T.CKI2.163, pBI35T.CKI2.162, pEL2omega35T.CKI2.163

and pEL2omega35T.CKI2.162 exhibited virescence and shoot formation on all of the medium, Z-0, Z-0.1 and Z-1.

5 All of the calli transformed with these vectors exhibited the most rapid shoot formation on Z-0 and Z-0.1 medium, which tended to be more rapid shoot formation than the calli transformed with the control vectors pBI35T and pEL2omega35T, with culturing at any cytokinin concentration.

10 These results indicate that a cytokinin response is elicited in the absence of cytokinins with overexpression of either a portion or the entire region of the CKI2 gene.

Example 4 Expression of CKI2 gene in plant bodies

15 Next, the effect of introducing the CKI2 gene on plant individuals was investigated. Preparation of the constructs for introduction into the plants was carried out in the same manner as Example 3.

20 Specifically, the CKI2 cDNA obtained in Example 2 was used as the template for amplification of the region corresponding from the 178th methionine to the stop codon of the CKI2 protein by PCR (Stratagene) using primer No.162 and primer No.177, and this was inserted downstream from the cauliflower mosaic virus 35S promoter of the binary vector.

25 After confirming the nucleotide sequences of the prepared constructs, all of them included mutations and therefore the regions containing the mutated nucleotides were removed by digestion with restriction enzymes ScaI and SacI, and the mutations were repaired by ligation using the corresponding ScaI and SacI restriction enzyme fragments from the genomic clone obtained in Example 2. 30 The clone prepared in this manner was designated as pTK031.

35 Next, the vacuum infiltration method (Araki, T., Sokubutsu Saibou Kogaku [Plant Cell Engineering] Series 4, Model Plant Experiment Protocols, p.109-113, Shujunsha Publishing) was used for introduction of pTK031 or a

binary vector with no insert into *Arabidopsis thaliana*, and the obtained seeds were disseminated in agar medium containing kanamycin as a selectable marker for the transformants.

5 The medium was incubated for 12 days at 22°C under light condition and observed. At this stage, none of the 19 calli transformed with the vector alone formed scapes, but scape elongation was found in 11 of the 45 calli transformed with pTK031.

10 In the case of *Arabidopsis thaliana*, scape elongation is accompanied by Horal meristem formation, and therefore these results demonstrated that introduction of pTK031 hastened the period of shifting from vegetative growth to reproductive growth.

15 It was thus shown that in transformants in which a portion of the CKI2 gene is cloned under the cauliflower mosaic virus 35S promoter, transition from vegetative growth to reproductive growth occurs much more rapidly. Consequently, by using all or a portion of the CKI2 gene
20 it is possible to artificially regulate the Horal formation period.

Industrial Applicability

25 In the manner described above, activation tagging was carried out to isolate the CKI2 gene coding for a histidine protein kinase involved in the cytokinin signal transduction pathway from *Arabidopsis thaliana*. In cki2 mutants, a cytokinin response including cell division and adventitious shoot formation was exhibited even in the
30 absence of cytokinins due to constitutive synthesis of CKI2 protein lacking the N-terminal region. It is therefore clear that the cytokinin response can be controlled by controlling expression of the CKI2 gene, and thus by controlling expression of the CKI2 gene, it
35 has become possible to control the various physiological effects exhibited by cytokinins in plants.

CLAIMS

1. A gene coding for a protein having the amino acid sequence listed as SEQ. ID. No.2, and involved in cytokinin signal transduction.
- 5 2. A gene coding for a protein having the amino acid sequence listed as SEQ. ID. No.2 modified with one or more amino acid additions, deletions and/or substitutions with other amino acids, and involved in cytokinin signal transduction.
- 10 3. A gene which hybridizes with nucleic acid having the nucleotide sequence listed as SEQ. ID. No.1, or a portion thereof, under stringent conditions and which codes for a protein involved in cytokinin signal transduction.
- 15 4. A gene according to any one of claims 1 to 3, wherein said protein is a histidine protein kinase.
5. A vector containing a gene according to any one of claims 1 to 4.
- 20 6. A host transformed by a vector according to claim 5.
7. A protein encoded by a gene according to any one of claims 1 to 4.
8. A method of producing a protein with histidine protein kinase activity, characterized in that a host according to claim 6 is cultured or cultivated and said protein is obtained from said host.
- 25 9. A plant or plant cells having introduced therein a gene according to any one of claims 1 to 4.
10. A method of regulating growth of a plant or plant cells, whereby a gene according to any one of claims 1 to 4 is introduced into a plant or plant cells and said gene is expressed.
- 30 11. A method of inducing adventitious shoot formation in a plant or plant cells, whereby a gene according to any one of claims 1 to 4 is introduced into a plant or plant cells and said gene is expressed.
- 35 12. A method of regulating the Horal formation

period of a plant, whereby a gene according to any one of claims 1 to 4 is introduced into a plant or plant cells and said gene is expressed.

ABSTRACT

5 There is provided a gene coding for a protein
involved in cytokinin signal transduction having the
amino acid sequence listed as SEQ. ID. No.2, which is
derived, for example, from *Arabidopsis thaliana*. The
gene may be utilized for production of the protein, or it
may be introduced into a plant for plant regeneration,
differentiation, growth or the like.

20250706 10069301.0000

Fig.1

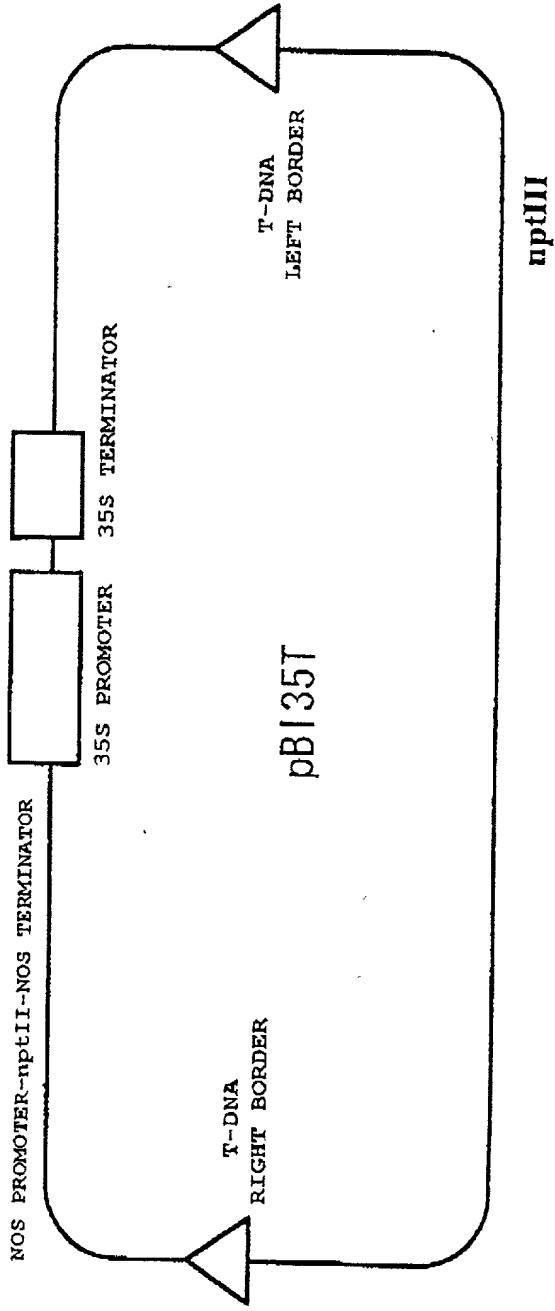


Fig. 2

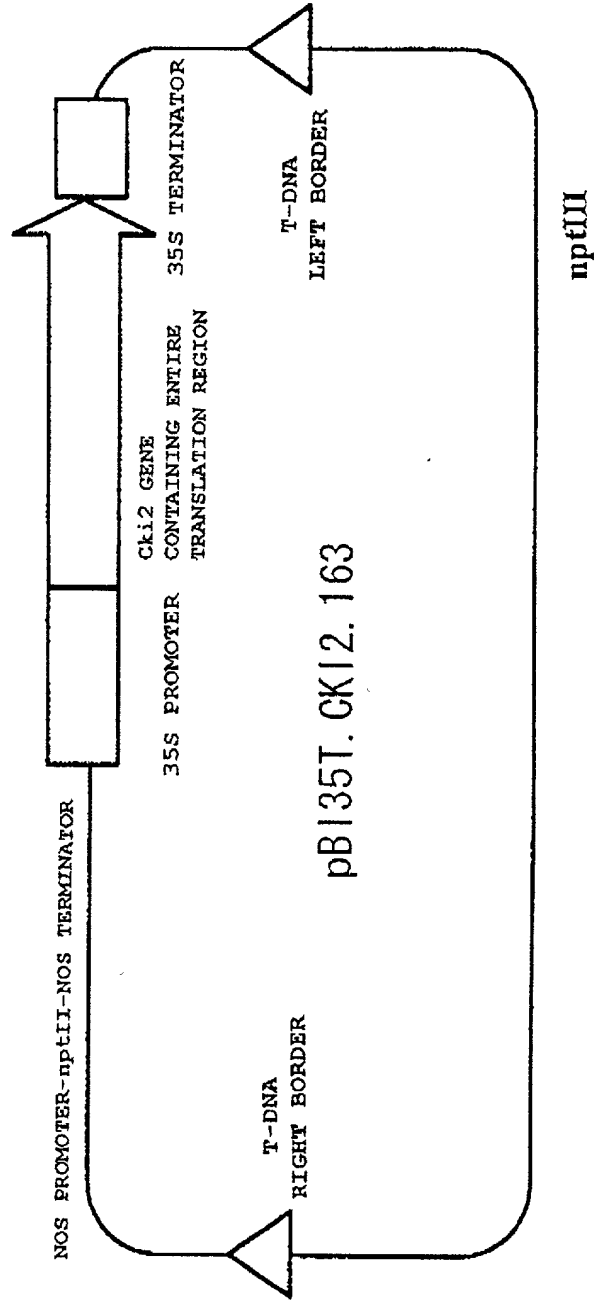


Fig. 3

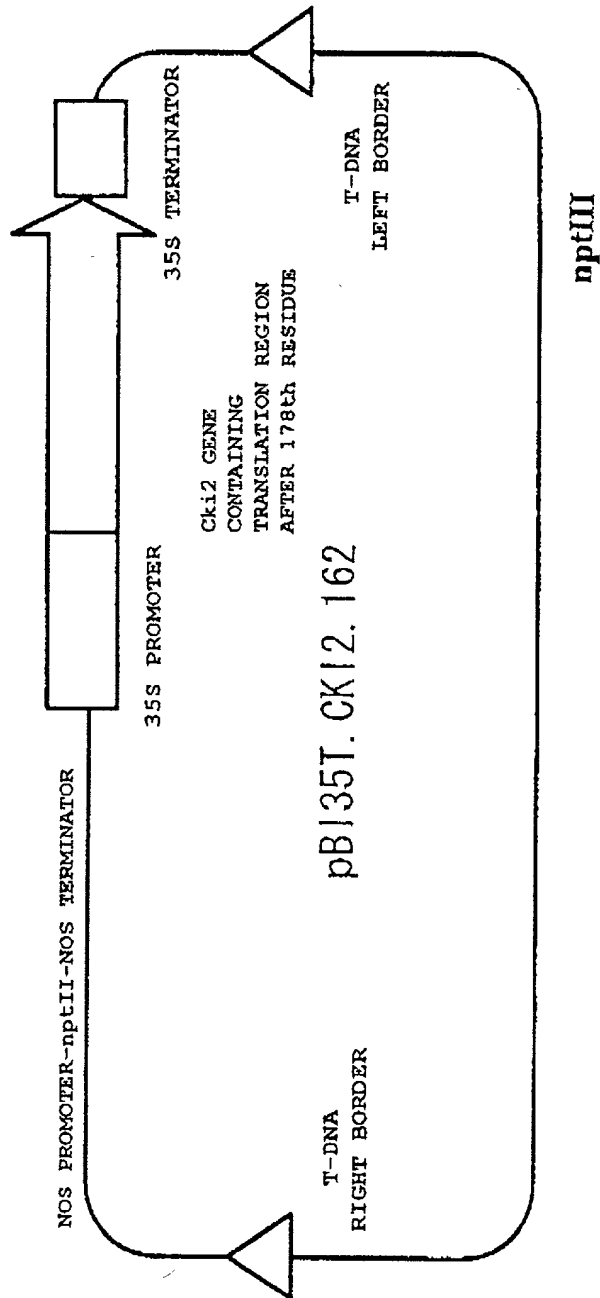


Fig. 4

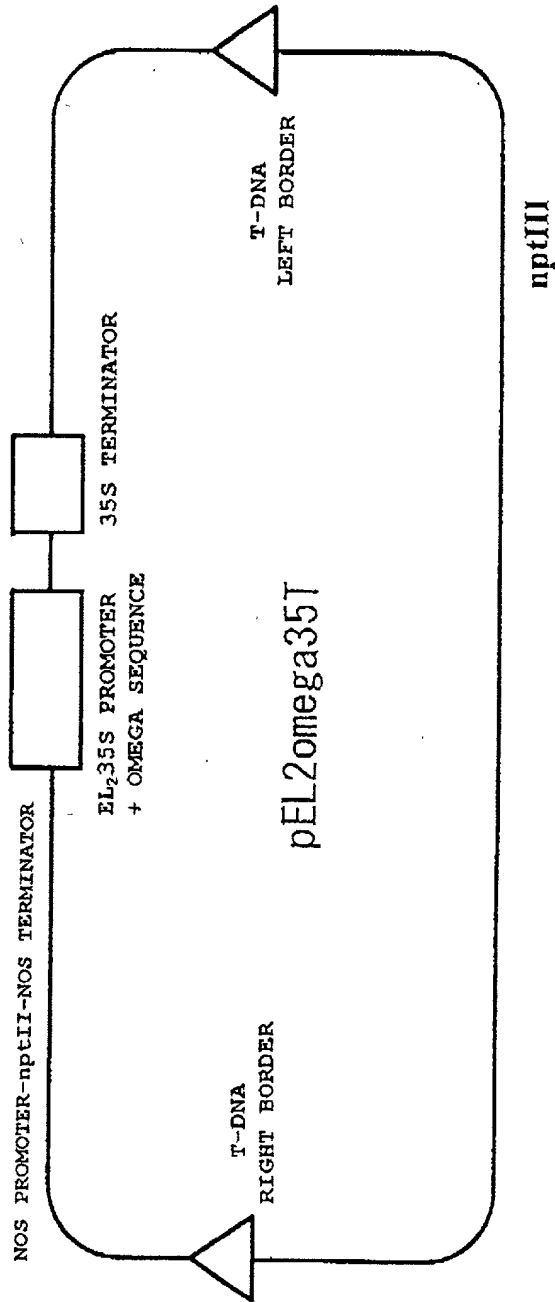


Fig. 5

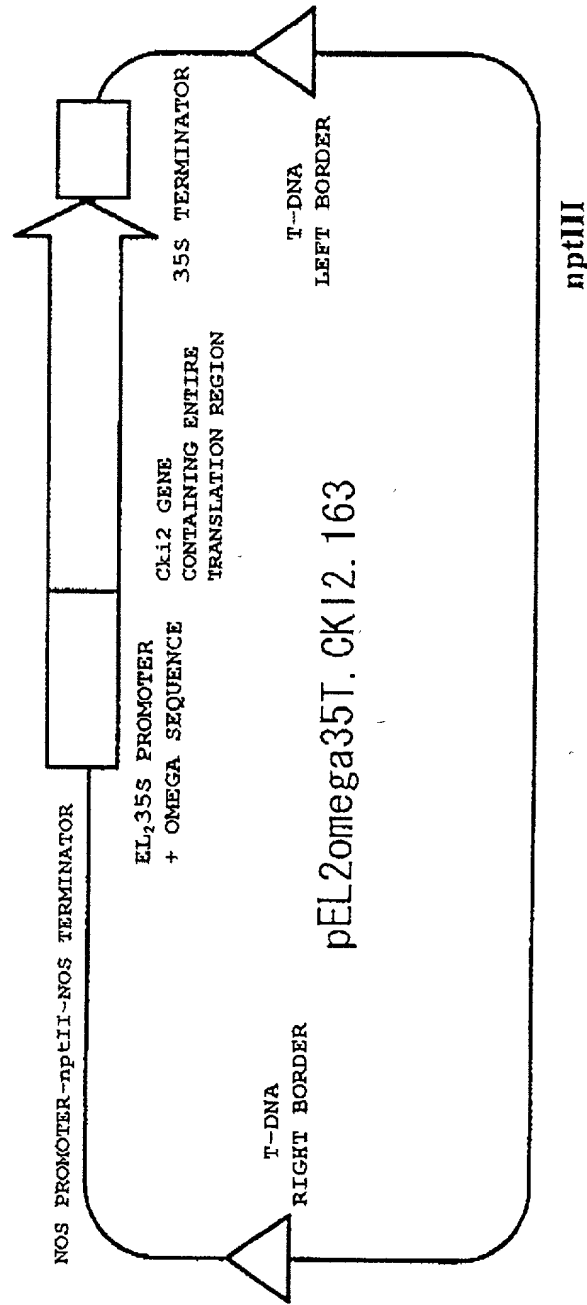
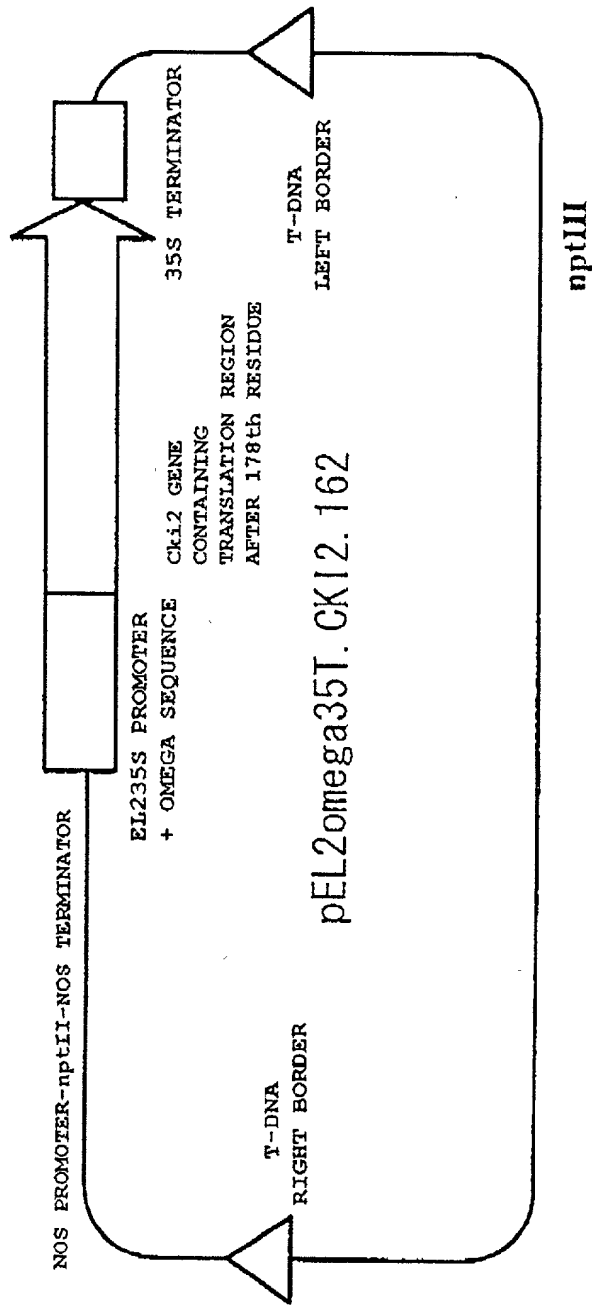


Fig. 6



Declaration and Power of Attorney For Patent Application

特許出願宣言書及び委任状

Japanese Language Declaration

日本語宣言書

下記の氏名の発明者として、私は以下の通り宣言します。

As a below named inventor, I hereby declare that:

私の住所、私書箱、国籍は下記の私の氏名の後に記載された通りです。

My residence, post office address and citizenship are as stated next to my name.

下記の名称の発明に関して請求範囲に記載され、特許出願している発明内容について、私が最初かつ唯一の発明者（下記の氏名が一つの場合）もしくは最初かつ共同発明者であると（下記の名称が複数の場合）信じています。

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

GENE CODING FOR PROTEIN INVOLVED IN

CYTOKININ SIGNAL TRANSDUCTION

上記発明の明細書（下記の欄でx印がついていない場合は、本表に添付）は、

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☐ 月 日に提出され、米国出願番号または特許協定条約国際出願番号を _____ とし、
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☒ was filed on August 25, 2000
as United States Application Number or
PCT International Application Number
PCT/JP00/05772 and was amended on
_____ (if applicable).

私は、特許請求範囲を含む上記訂正後の明細書を検討し、内容を理解していることをここに表明します。

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

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I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

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私は、米国法典第35編119条(a)-(d)項又は365条(b)項に基づき下記の、米国以外の国の少なくとも一カ国を指定している特許協力条約365(a)項に基づき国際出願、又は外国での特許出願もしくは発明者証の出願についての外国優先権をここに主張するとともに、優先権を主張している、本出願の前に出願された特許または発明者証の外国出願を以下に、枠内をマークすることで、示しています。

Prior Foreign Application(s)

外国での先行出願

11-240433(Pat.Appln.)

(Number)
(番号)

Japan

(Country)
(国名)

August 26, 1999

(Day/Month/Year Filed)
(出願年月日)

Priority Not Claimed

優先権主張なし

☐

(Number)
(番号)

(Country)
(国名)

(Day/Month/Year Filed)
(出願年月日)

☐

私は、第35編米国法典119条(e)項に基づいて下記の米国外特許出願規定に記載された権利をここに主張いたします。

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

(Application No.)
(出願番号)

(Filing Date)
(出願日)

(Application No.)
(出願番号)

(Filing Date)
(出願日)

私は、下記の米国法典第35編120条に基づいて下記の米国外特許出願に記載された権利、又は米国を指定している特許協力条約365条(c)に基づき権利をここに主張します。また、本出願の各請求範囲の内容が米国法典第35編112条第1項又は特許協力条約で規定された方法で先行する米国外特許出願に開示されていない限り、その先行米国外出願を提出日以降で本出願書の日本国内または特許協力条約国際提出日までの期間中に入手された、連邦規則法典第37編1条56項で定義された特許資格の有無に関する重要な情報について開示義務があることを認識しています。

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of application.

(Application No.)
(出願番号)

(Filing Date)
(出願日)

(Status: Patented, Pending, Abandoned)
(現況: 特許許可済、係属中、放棄済)

(Application No.)
(出願番号)

(Filing Date)
(出願日)

(Status: Patented, Pending, Abandoned)
(現況: 特許許可済、係属中、放棄済)

私は、私自身の知識に基づいて本宣言書中で私が行なう表明が真実であり、かつ私の入手した情報と私の信じることに基づき表明が全て真実であると信じていること、さらに故意になされた虚偽の表明及びそれと同等の行為は米国法典第18編第1001条に基づき、罰金または拘禁、もしくはその両方により処罰されること、そしてそのような故意による虚偽の表明を行なえば、出願した、又は既に許可された特許の有効性が失われることを認識し、よってここに上記のごとく宣誓を致します。

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Japanese Language Declaration (日本語宣言書)

委任状: 私は下記の発明者として、本出願に関する一切の
手続を米特許商標局に対して遂行する弁理士または代理人
として、下記の者を指名いたします。(弁理士、または代理
人の氏名及び登録番号を明記のこと)

POWER OF ATTORNEY: As a named inventor, I hereby appoint
the following attorney(s) and/or agent(s) to prosecute this
application and transact all business in the Patent and Trademark
Office connected therewith (list name and registration number)

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Benton S. Duffett, Jr.	22,030	R. Danny Huntington	27,903	Michael G. Savage	32,596
Joseph R. Magnone	24,239	Eric H. Weisblatt	30,505	Gerald F. Swiss	30,113
Norman H. Stepno	22,716	James W. Peterson	26,057	Michael J. Ure	33,089
Ronald L. Grudziecki	24,970	Teresa Stanek Rea	30,427	Charles F. Wieland III	33,096
Frederick G. Michaud, Jr.	26,003	Robert E. Krebs	25,885	Bruce T. Wieder	33,815
Alan E. Kopecki	25,813	William C. Rowland	30,888	Todd R. Walters	34,040
Regis E. Sluiter	26,999	T. Gene Dillehunt	25,423		
Samuel C. Miller, III	27,360	Patrick C. Keane	32,858		
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日付

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Residence

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日付

Second inventor's signature

Date

住所

Residence

国籍

Citizenship

私書箱

Post Office Address

(第三以降の共同発明者についても同様に記載し、署名をす
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(Supply similar information and signature for third and subsequent
joint inventors.)

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 Gln Phe Asn Val Glu Lys Pro Ala Gly Asp Leu Asp Thr Leu Lys Glu
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Glu Ser Glu Asp Phe Lys Arg Glu Val Leu Glu Lys Gly Lys Ala Ser	
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Lys Arg Glu Ile Thr Phe Thr Thr Asp Leu Phe Gly Ser Lys Thr Phe	
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Leu Ile Tyr Val Glu Pro Val Tyr Asn Lys Ala Gly Glu Lys Ile Gly	
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Val Val Lys Arg Glu Lys Met Ala Lys Leu Arg Glu Asp Asn Ala	
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Val Arg Lys Ala Met Glu Ser Glu Leu Asn Lys Thr Ile His Ile Thr	
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agacttaggt gttgtgtttt ttottttgca g ag gag aca atg aga gca aag cag	1440
Glu Glu Thr Met Arg Ala Lys Gln	
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Met Leu Ala Thr Met Ser His Glu Ile Arg Ser Pro Leu Ser Gly Val	
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 Val Gly Met Ala Glu Ile Leu Ser Thr Thr Lys Leu Asp Lys Glu Gln
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 Arg Gln Leu Leu Asn Val Met Ile Ser Ser Gly Asp Leu Val Leu Gln
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 Leu Ile Asn Asp Ile Leu Asp Leu Ser Lys Val Glu Ser
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 Gly Val Met Arg Leu Glu Ala Thr
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 Asn Ala Ile Lys Phe Thr His Glu Gly Asn Val Gly Ile Lys
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 Leu Gln Val Ile Ser Glu Pro Ser Phe Val Arg Asp Asn Ala Leu Asn
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 Leu Pro Tyr Lys Val Gly Arg Ser Asp Asp Tyr Ser Asp Asp Gln Asp
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 Thr Ser Pro Ile Lys Leu Ile Asn Gly Phe Val Ala Asp Pro Ser Asn
 675 680 685
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725 730 735
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755 760 765
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770 775 780 785
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Asn Ser Ser Ser Tyr Asp Leu Val Leu Met
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Arg Pro Thr Asn Arg Leu Pro Ile Ile Ala
875 880

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Tyr Ala Asn Gly Met Asp Ser Phe Ile Ser Lys Pro Val Thr Leu Gln

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Gln Phe Asn Val Glu Lys Pro Ala Gly Asp Leu Asp Thr Leu Lys Glu

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Val Thr Ile Glu Thr Arg Thr Ile Ala Asp Met Thr Arg Leu Pro Asn

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Leu Leu Asn Ser Thr His Gln Gly Ser Ser Gln Leu Thr Asn Leu Val

65 70 75 80

Lys Gln Trp Glu Tyr Met Gln Asp Asn Ala Val Arg Leu Leu Lys Glu

85 90 95

Glu Leu Lys Asn Leu Asp Arg Gln Arg Glu Glu Ala Glu Ala Lys Glu

100 105 110

Leu Lys Ile Ile Glu Glu Tyr Lys Phe Glu Ser Asn Glu Pro Glu Asn

115 120 125

Val Pro Val Leu Asp Glu Thr Ser Asp Leu Phe Arg Arg Phe Arg Gln

130 135 140

Lys Lys Arg Asp Ala Leu Val Asp Ser Lys Lys Ile Glu Ile Tyr Glu

145 150 155 160

Glu Phe Asp Thr Val Ala Tyr Trp Lys Gln Lys Ala Leu Ser Leu Glu

165 170 175

Lys Met Leu Glu Ala Ser Thr Glu Arg Glu Arg Arg Leu Met Glu Lys
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 Glu Leu Thr Gln Asn Leu Lys Arg Ala Glu Gly Phe Leu His Phe Ile
 210 215 220
 Leu Gln Asn Ala Pro Ile Val Met Gly His Gln Asp Lys Asp Leu Arg
 225 230 235 240
 Tyr Leu Phe Ile Tyr Asn Lys Tyr Pro Ser Leu Arg Glu Gln Asp Ile
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 Leu Gly Lys Thr Asp Val Glu Ile Phe His Gly Gly Gly Val Lys Glu
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 Ser Glu Asp Phe Lys Arg Glu Val Leu Glu Lys Gly Lys Ala Ser Lys
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 Arg Glu Ile Thr Phe Thr Thr Asp Leu Phe Gly Ser Lys Thr Phe Leu
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 Ile Tyr Val Glu Pro Val Tyr Asn Lys Ala Gly Glu Lys Ile Gly Ile
 305 310 315 320
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 Met Ala Lys Leu Arg Glu Asp Asn Ala Val Arg Lys Ala Met Glu Ser
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 Gln Met Leu Ala Thr Met Ser His Glu Ile Arg Ser Pro Leu Ser Gly
 370 375 380
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 Gly Asn Ile Ala Asp Asp Val Pro Ile Glu Val Val Gly Asp Val Leu
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Arg Ile Arg Gln Ile Leu Thr Asn Leu Ile Ser Asn Ala Ile Lys Phe
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 Thr His Glu Gly Asn Val Gly Ile Lys Leu Gln Val Ile Ser Glu Pro
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 Ser Phe Val Arg Asp Asn Ala Leu Asn Ala Asp Thr Glu Glu His Glu
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 Asp Thr Gly Ile Gly Ile Pro Glu Asn Ala Leu Pro Cys Leu Phe Lys
 545 550 555 560
 Lys Tyr Met Gln Ala Ser Ala Asp His Ala Arg Lys Tyr Gly Gly Thr
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 Gly Leu Gly Leu Ala Ile Cys Lys Gln Leu Val Glu Leu Met Gly Gly
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 Gln Leu Thr Val Thr Ser Arg Val Asn Glu Gly Ser Thr Phe Thr Phe
 595 600 605
 Ile Leu Pro Tyr Lys Val Gly Arg Ser Asp Asp Tyr Ser Asp Asp Gln
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 690 695 700
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 Ala His Gln Tyr Glu Asn Gly Asn Gly Arg Cys Phe Ser Lys Glu Ser
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 Glu Ser Cys Ser Ser Ser Gln Ala Ser Ser Glu Gly Gly Thr Leu Glu
 740 745 750
 Met Glu Ser Glu Leu Thr Val Ser Ser His Arg Glu Glu Glu Lys Ala
 755 760 765
 Glu Ile Glu Val Lys Glu Thr Ser Lys Pro Asn Ile Leu Leu Val Glu
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Asp Asn Lys Ile Asn Ile Met Val Ala Lys Ser Met Met Lys Gln Leu
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 Gly His Thr Met Asp Ile Ala Asn Asn Gly Val Glu Ala Ile Thr Ala
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